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NF-κB/Rel exists in non-B cells in inactive forms sequestered in the cytoplasm by IκBs, but can be induced by oxidative status. We discovered that breast cancer is typified by aberrant constitutive expression of NF-κB/Rel. The incidence of breast cancer is low in areas of the world where green tea consumption is high. Green tea contains compounds such as epigallocatechin 3-gallate (EGCG) which possess anti-oxidant properties. Using a cell proliferation assay, we found that growth of Hs578T breast cancer cells was slowed at 20-80 ng/ul EGCG, while at higher doses (160 ng/ul) cell numbers decreased. This decrease was due to apoptosis, as assessed by trypan blue and TUNEL assays, respectively. EGCG similarly inhibited growth of other cell lines: D3-1, a carcinogen-transformed clonal cell line of untransformed MCF 10F cells, and MDA-MB-231, a human cancer cell line derived from a patient with breast adenocarcinoma. To test the role of NF-κB in mammary tumorigenesis, we prepared a transgenic mouse model, which overexpresses a dominant negative version of IκB-α. This gene was targeted to the mammary gland using the mouse mammary tumor virus (MMTV-LTR) promoter. Transgenic founder lines have been confirmed by Southern blot. To determine transgene expression levels Northern blot analysis are being done.

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Kathryn Kavanagh 5/10/00

PI - Signature Date

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INTRODUCTION

The incidence of breast cancer has been steadily rising over the past 50 years, and is now one of the leading causes of death among American women between the ages of 40-55 (27). In an attempt to elucidate the reasons for this increase in incidence, both genetic and environmental factors are being investigated. Recently, it has become apparent that the bioaccumulation of environmental pollutants may be a contributing factor of notable significance (17, 23, 43, 65). The polycyclic aromatic hydrocarbons (PAH's) such as 7, 12-dimethylbenz(α)-anthracene (DMBA) are of particular interest (43). One of the earliest events in PAH tumorigenesis is the binding of the carcinogen to a cytosolic aromatic hydrocarbon receptor (AhR) (15, 22, 50). The receptor-ligand complex is subsequently translocated to the nucleus where it can bind to and alter the transcriptional level of DNA that contains AhR-responsive elements. The Phase I cytochrome P-450 family of enzymes is classically induced as a result of exposure to PAH's (14, 35, 43, 45, 68). These enzymes play a major role in the detoxification of harmful substances in the body, such as chemical carcinogens and drugs, by a process of oxidative metabolism. Often, reactive oxygen species (ROS) are produced during these metabolic reactions (68). Reactive oxygen species are potentially harmful substances which are capable of causing DNA damage, and thus place the cell under considerable oxidative stress. In addition, we hypothesize that the NF–κB/Rel family of transcription factors may be induced as a result of increased oxidative stress caused by environmental pollutants. This family of factors regulates transcription of multiple genes, including some such as the c-*myc* oncogene involved in the regulation of cell proliferation, neoplastic transformation (21, 31, 34) and cell survival (7, 37, 62, 64, 66). NF-κB/Rel factor activity has been found to be sensitive to the redox state of the cell. Our recent data indicate that malignant breast cancer cell lines and primary breast cancer tissue express significant levels of constitutive nuclear NF-κB/Rel activity (56). Furthermore, we have shown that microinjection of IκB-α protein into 578T cells in vitro produces apoptosis in more than 20% of the cells. Thus, we hypothesize that constitutive expression of this factor suggests that NF-κB/Rel may promote aberrant proliferation and/or cell survival and thus play an early role in the pathogenesis of breast tumors.

NF-κB/Rel Family of Transcription Factors

The NF-κB/Rel family of transcription factors was first identified as a protein family constitutively expressed specifically in mature B lymphocytes. NF-κB/Rel subunits are known to interact with the B site of the kappa light (L) chain gene enhancer (54). In most non-B cells, inactive NF-κB protein is present sequestered in the cytoplasm complexed with inhibitory proteins, called IκBs (4). Activation and nuclear localization can be induced by several agents, including oxidative stress (reviewed in references 5, 9, 25). We and others have shown that activation of NF-κB is achieved by phosphorylation of IκB which is then degraded via the proteasome pathway (13, 28), upon which active NF-κB dimers translocate to the nucleus. There they bind to κB-responsive elements, causing upregulation of certain gene products. We demonstrated that the murine c-*myc* oncogene contains two functional κB sites (20, 32). The human c-*myc* gene was found to contain similar κB sites. To date, NF-κB has been implicated in the transcriptional regulation of a number of cellular genes involved in the control of cell proliferation, adhesion and in immune and inflammatory responses (5, 9, 24). These include the oncogene c-*myc*, several genes encoding growth factors or interleukins or their receptors, and adhesion molecules such as E-selectin, ICAM-1 and VCAM-1.

The biochemical characterization of classical NF-κB determined that it is a heterodimer composed of a 50kDa (p50) and a 65 kDa (p65) subunit. The binding domains of these factors have homology with an approximate 300 amino acid domain of the v-Rel oncoprotein and was thus termed the Rel homology domain (RHD), hence this family is termed the Rel or NF-κB/Rel family (24, 33, 48). In addition to c-Rel, other members of the mammalian Rel family have been discovered, including p52 (also called lyt10) (12, 46), RelB (49). Rel-related factors bind as hetero- or homodimers that have different activities depending on subunit composition (61). For example, the p65 subunit is able to potently transactivate promoters driven by κB elements (34, 52). The c-Rel protein, which appears to function in an element specific fashion, transactivates more moderately (34, 36, 41, 58). RelB is also a potent transactivator but only functions as a heterodimer (19, 49). The overall effect within a cell is determined by the balance of dimers expressed, and is specific to the gene of interest. Our preliminary results indicate malignant breast cell lines and primary tissue constitutively express active Rel factors, including c-Rel and p65.

NF-κB Inhibitory Proteins

NF-κB/Rel dimers are maintained in an inactive state in the cytoplasm bound to members of the IκB family of inhibitory proteins. Family members include IκB-α (MAD-3), IκB-β, IκB-ε, p105 and p100, each of which contains conserved motifs called ankyrin repeats, through which they associate with NF-κB proteins (24). The IκBs act by blocking the nuclear localization signal of the NF-κB dimer, preventing its translocation to the nucleus. As discussed above, these proteins are phosphorylated on specific serine residues, which leads to their ubiquitination and subsequent degradation via the proteasome pathway (13, 16).

Aberrant Constitutive Expression of NF-κB/Rel factors in Breast Cancer

Our laboratory found that nuclear extracts form two untransformed breast epithelial cell lines contained only low, basal levels of specific binding in electromobility shift analysis (EMSA) using a labeled NF-κB oligonucleotide as probe. In contrast, extracts prepared from the malignant cells had much higher levels of binding. We have also shown that NF-κB/Rel subunits are active, i.e., nuclear, in multiple primary human tumor tissue specimens (56). Furthermore, we demonstrated this activity provided survival signals to the breast cancer cells. Specifically, we have shown that microinjection of IκB-α protein or of an affinity purified antibody to the NF-κB/Rel subunit expressed in 578T breast cancer cells in culture produced apoptosis in greater than 20% of the cells (56).

The DMBA Rat Model of Breast Cancer

Rat models for breast cancer are widely used. The DMBA single dose model has permitted elucidation of factors that act at initiation of neoplasia in the gland and at the subsequent steps on tumor development. Rats show maximum sensitivity to DMBA tumorigenesis in the period beginning shortly after vaginal opening and ending after establishment of regular estrous cycles. This is a period of rapid growth of the gland and occurs between approximately 35 and 60 days of age. Increasing age, completed pregnancy and lactation all decrease susceptibility of the gland to chemical carcinogenesis and increase the proportion of benign tumors. Treatment of female Sprague-Dawley rats with a single dose of the polycyclic aromatic hydrocarbon DMBA results in the induction of mammary gland (breast) tumors within 7-20 weeks.

In our animal study, rats were administered either 15 mg/kg DMBA (in sesame oil) or carrier sesame oil alone, as control (5 animals per condition). Nuclear extracts prepared individually from combined mammary gland preparations from each animal. EMSA analysis was performed with the URE, as probe.

Protective effects of Green Tea

Epidemiological studies suggest that green tea consumption may have a protective effect against tumor formation (29, 44, 67). Statistics indicate that the incidence of breast cancer in regions where green tea is consumed in large quantities, including China and Japan, is much lower than in western society. Furthermore, in our preliminary studies using the DMBA rat model, we have found green tea can reduce the resulting mammary tumor burden, consistent with the results of Hirose, et al., 1994 (29). It is known that green tea contains several compounds which possess anti-oxidant qualities. These compounds are the Green Tea Polyphenols (GTP), which include epicatechin, epicatechingallate epigallocatechin and epigallocatechin-3 Gallate (EGCG). Concentrated, purified polyphenol extracts of green tea have anticarcinogenic activity. The toxicity of these extracts is low and they are potentially important cancer chemopreventive agents. We hypothesize that the anti-oxidant activity of green tea provided by the GTPs counteracts the oxidative stress which arises as a result of cellular metabolism. A reduction in oxidative stress would in turn lower NF-κB/Rel levels, a key step in the prevention of tumor formation.

BODY AND CONCLUSIONS

Proposed Aims

Aim 1: Determine the effects of green tea on breast cancer cell lines.

Growth curves

To determine whether treatment with green tea extracts decreases the growth of breast cancer cells *in vitro*, Hs578T breast cancer cells were treated with concentrations of green tea polyphenols (GTP) or epigallocatechin-3 Gallate (EGCG) ranging from 0-160 ng/ul. Cell growth was assessed by cell numbers using a hemocytometer and by MTS assay. Studies, performed over a 72 hours treatment period, showed that GTP slowed the growth of the Hs578T breast cancer cells (Fig. 1). This effect was exhibited in a dose-dependent fashion, with lower doses (20-40 ng/ul) of GTP slowing growth, while higher doses (100-160 ng/ul) causing a decrease in cell numbers. Our cell counting data were supported by results obtained using the MTS assay of cell viability (Fig. 2).

We next turned our attention to EGCG, the most potent known polyphenolic component of green tea. Cell counting experiments performed on Hs578T cells using EGCG solution revealed that it too slowed the growth of the cells in a dose-dependent fashion (Fig. 3). At lower concentrations (below 40 ng/ul) of EGCG, we observed slowed cell growth, while higher dosages (80-160 ng/ul) caused a decline in cell numbers. A lower concentration of 40 ng/ul of EGCG was required to exert the same effects as 80 ng/ul of GTP.

Trypan Blue Assay

To determine whether the Hs578T cells were undergoing growth arrest or cell death, trypan blue exclusion analyses were performed. To ensure that dead, floating cells were not lost during the preparation, all media were collected and added back to the final cell volume. At dosages of 80

ng/ul, cells did not take up the trypan blue dye, indicating that they were viable. In contrast, at 160 ng/ul of EGCG a significant level of death was clearly visible (Fig. 4). Therefore, EGCG is capable of killing cancer cells *in vitro* at a dose higher than those needed to arrest growth.

TUNEL Assay

Next we wanted to determine whether the death seen at the higher doses of EGCG treatment was due to apoptosis. To this end, a TUNEL assay was performed. At 40 ng/ul of EGCG, no significant apoptotic index was recorded. However, at concentrations of 80 ng/ul, death by apoptosis was clearly detected (Fig. 5). Taken together, these findings indicate that EGCG slows growth at 40 ng/ul, while at 80 ng/ul, it causes irreversible growth arrest and apoptosis of Hs578T breast cancer cells.

Studies in other breast cancer cell lines

I next sought to determine whether the effects of EGCG could be extended to other estrogen receptor negative (ER-) breast cancer cell lines. The medical community is searching for adjuvants that may be effective against ER- breast tumors, as these tumors are not responsive to anti-estrogenic treatments such as tamoxifen, and generally indicate a poorer prognosis.
The MTS assay was performed on MDA-MB-231 and D3-1 breast cancer cells. The MDA-MB-231 cell line is a human cancer cell line derived from a patient with poorly differentiated adenocarcinoma of the breast. D3-1 is a carcinogen-transformed clonal cell line derived consequent to treatment of the untransformed MCF10F breast epithelial cell line with DMBA. MDA-MB-231 cells displayed a dose-response curve to EGCG treatment similar to that of Hs578T cells, indicating that these cells are also sensitive to the EGCG growth retardation effects (Fig. 6). The D3-1 cell line was more sensitive to the effects of EGCG. A dose of 40 ng/ul of EGCG caused the same amount of growth arrest in D3-1 cells as did 80 ng/ul in Hs578Tcells (Fig. 7). Furthermore, a significant level of apoptosis was detected at 80 ng/ul and 100% of the cells had undergone apoptosis at 160 ng/ul (Fig. 8). I next examined an ER+ cell line, MCF-7. Surprisingly, the MCF-7 cell line appeared more resistant to the effects of EGCG. Cell number continued to increase even at 80 ng/ul (Fig. 9).
Conclusions: From these results I conclude that the cellular response to EGCG treatment is somewhat cell-type specific, although the majority of cells treated display a growth inhibitory effect at low concentrations and an apoptotic response at higher concentrations.

Aim 2. Quantitate the relative levels of NF-κB/Rel subunit expression in normal and malignant mammary tissue from DMBA-treated rats +/- green tea treatment.

To study the NF-κB levels in mammary tissue from DMBA-treated rats, I first prepared nuclear extracts as follows:
Samples were pulverized on dry ice using a Bessman Tissue Pulverizer (Spectrum). Frozen tissue powder was homogenized in 0.5 g/ml TEGT/MO buffer (50 mM Tris/HCl, 1 mM EDTA, 10% glycerol, 10 mM monothioglycerol, 10 mM sodium molybdate pH 7.4 containing 0.02% sodium azide) using a Polytron (Kinematica, Luzern, Switzerland). After the first few seconds of homogenization, proteolytic inhibitors were added (0.5 mM PMSF, 1 ug/ml LP, 100 ug/ml aprotinin, 10 ug/ml pepstatin, and 100 ug/ml bacitracin). Homogenates were centrifuged for 10 min at 3,000 rpm at 2°C. The cytoplasmic supernatant was removed and the nuclear pellets were either stored at -80°C, or immediately washed once in plus DTT, PMSF and LP with

centrifugation in a clinical centrifuge for 5 min at setting 5 at 4°C. The nuclei were purified by passing through a sucrose gradient as follows: nuclei were gently resuspended in ice cold Buffer A (300 mM sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes pH 7.5, 2 mM EDTA, 14 mM β-mercaptoethanol) plus DTT, PMSF and LP. This suspension was gently layered over an equal volume of Buffer B (the same as Buffer A except for an increase in sucrose to 30%). This gradient was centrifuged at 2,500 rpm for 5 min at 4°C. The supernatant was gently aspirated and the nuclear pellet washed twice with cold hypotonic RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4) containing 0.5% NP-40 detergent plus DTT, PMSF, and LP as above. Following a 15 minute incubation on ice, the cells were dounce homogenized (pestle A) using 30-60 strokes, or until cell lysis occurred, as assessed by microscopic evaluation of an aliquot of cells stained with crystal violet. The homogenate was transferred to an eppendorf tube and centrifuged for 5 min at setting 5 at 4°C using a microcentrifuge. The nuclear pellet was washed once in RSB plus DTT, PMSF and LP. Nuclei were centrifuged as above, and resuspended in 1.5 packed nuclear volumes of extraction Dignam-Roeder Buffer (420 mM KCl, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) plus DTT, PMSF and LP as above (18), and incubated on ice for 30 min with occasional tapping. The extracted proteins were separated from the cellular debris by centrifugation in a Sorvall SA600 rotor for 5 min at 5,000 rpm at 4°C. The supernatant containing nuclear proteins was carefully removed and stored at -80°C. Protein concentration was determined using the Bio-Rad protein assay.

Samples of nuclear extracts were subjected to EMSA. For the binding reaction, ^{32}P-labeled oligonucleotide (20,000 - 25,000 cpm) was incubated with 5 ug of nuclear extract, 5 ul sample buffer (10 mM HEPES, 4 mM dithiothreitol, 0.5% Triton X-100, and 2.5% glycerol), 0.5 ug poly dI-dC as nonspecific competitor, the salt concentration adjusted to 100 mM using Dignam-Roeder buffer, and the final volume brought to 25 ul with water. The reagents were gently mixed and the reaction carried out at room temperature for 30 min. DNA/protein complexes were subjected to electrophoresis at 11 V/cm and resolved on a 4.5% polyacrylamide gel (using 30% acrylamide/0.8 % bisacrylamide) with 0.5x TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0).

Previous studies indicated that histologically normal breast tissue derived from female Sprague-Dawley rats had low levels of NF-κB binding (Sovak et al., 1997). Tumor tissue derived from rats treated with DMBA and subsequently fed water displayed significantly higher levels of NF-κB binding (Fig. 10), as seen previously (Sovak et al., 1997). Interestingly tumor samples from rats that were fed green tea also showed high levels of NF-κB, essentially comparable to those fed water (Fig. 10). This result may be interpreted that tumor cells that had low levels of NF-κB were not allowed to develop. Alternatively, it may be that tumor cell with high NF-κB levels better survive the green tea treatment. Consistent with these two possibilities, the tumors from rats fed green tea were significantly smaller than those from the untreated group, with an overall decrease in tumor burden of 70%. On the other hand, it is possible that green tea inhibition of cell proliferation is not mediated via affects on NF-κB levels.

Conclusions and future directions: I will investigate which subunits of NF-κB/Rel are overexpressed in the malignant mammary tissue by western blot analysis.

Aim 3. Test the effects of inducible expression of IκB-α in breast cancer cell lines and in nude mice.

With help from Greg Zanieski, I constructed an dnIκB-α Hs578T cell line under the control of the lacZ repressor. Unfortunately, when these clones were analyzed, the basal expression of NF-κB was greatly reduced. Leaky expression from these "inducible" vector system has proven to be a serious problem for many investigators. Therefore, since I had already made significant progress in the preparation of the MMTV-dnIkB-α mouse, we decided to focus on this approach to this question, which is described in Aim 4, below. We now propose to use retroviral infection to investigate the effects of IκB-α expression in the breast cancer cell lines. To this end, retroviral expression vectors have been constructed in our lab. Our transgenic mice studies are proceeding quite well, however, and perhaps our results with the *in vivo* work may, in fact, eliminate the need to perform the breast cancer cell line experiments.
Future directions: We will use the retroviral infection procedure to investigate the effects of inducible expression of IκB-α in breast cancer cell lines and in nude mice.

Aim 4. Use transgenic mice to test protection against tumor formation when IκB-α is overexpressed.
The transgenic mouse has proven to be a useful tool for investigation into the involvement of many oncogenes in the pathogenesis of neoplasias. Several tumors have been described to date in transgenic mice, with particular attention being paid to tumors of the breast. By using a breast tissue-specific promoter/enhancer element such as the mouse mammary tumor virus long terminal repeat (MMTV-LTR) or a milk protein promoter, expression of a particular gene can be targeted selectively to the mammary gland. Several transgenic mice have been produced in this fashion, including mice with breast specific expression of *ras* (39, 59), *int-1* (60), c-*myc* (57), c-*neu* (38), and TGF-α (40, 55).

Creation of MMTV-IκB-α 32/36 A Transgenic Mice
Using the pRc/β-Actin vector containing a cDNA of a mutant form of IκB-α kindly donated by Joseph DiDonato and Michael Karin, I have created a recombinant plasmid vector which contains the IκB-α 32/36 A cDNA under the control of the MMTV promoter. This mutant is termed IκB-α 32/36 A because it contains serine to alanine mutations at amino acids positions 32 and 36, which prevent phosphorylation by IKK's and subsequent degradation of IκB-α. This vector will allow us to analyze the protective effects of constitutive expression of IκB-α in mice. The microinjections of mice have been performed by Daniel Ladd in the Core Transgenic Laboratory. Three potential female founders were identified by Southern Blotting. Founder number 36 seems to have incorporated the most copies of the transgene, while founders number 14 and 15 have an approximately equal number of transgene copies. I have bred founder mice with wild type male mice and assessed their offspring for germline transmission of the transgenes by Southern blotting of tail DNA (Fig. 11) and data not shown. As probe, I used a radioactively-labelled fragment of the construct released after digestion with Bam H1 restriction enzyme which contains the dominant negative IκB-α insert. Mammary gland extracts from pregnant founder mice demonstrate decreased endogenous, constitutive NF-κB binding, consistent with transgene expression (Fig. 12).

Conclusions and future directions: We are currently investigating the development of the normal mammary gland, and are preparing to treat these mice with carcinogen as outlined in the proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that EGCG slows the growth of Hs578T cells at low concentrations, as determined by MTS assay.
- Showed that at higher concentrations, EGCG causes apoptosis of breast cancer cell lines as demonstrated by trypan blue exclusion and TUNEL assays.
- Showed that tumor tissue derived from Sprague-Dawley rats treated with DMBA and subsequently fed water displayed high levels of NF-κB binding.
- Showed that tumors derived from rats treated with green tea also displayed high levels of NF-κB binding.
- Prepared MMTV-IκB-α 32/36A transgenic mice for *in vivo* study of the protective effect of IκB-α against the development of breast cancer.

REPORTABLE OUTCOMES

Mouse lines:

Prepard MMTV-IκB-α 32/36A transgenic mice for *in vivo* study of the protective effect of IkB-a against the development of breast cancer.

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Green Tea Polyphenol treatment of 578T cells

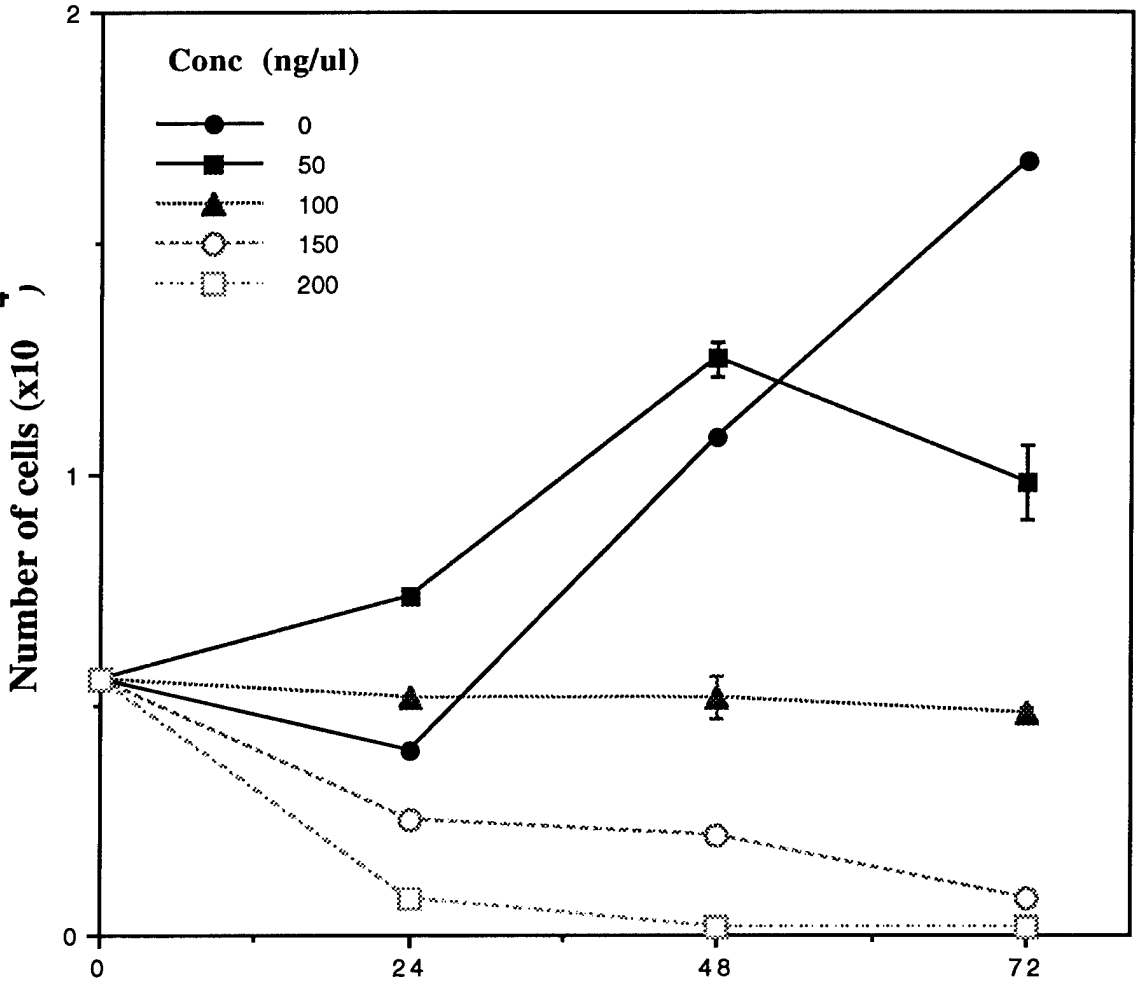


Figure 1

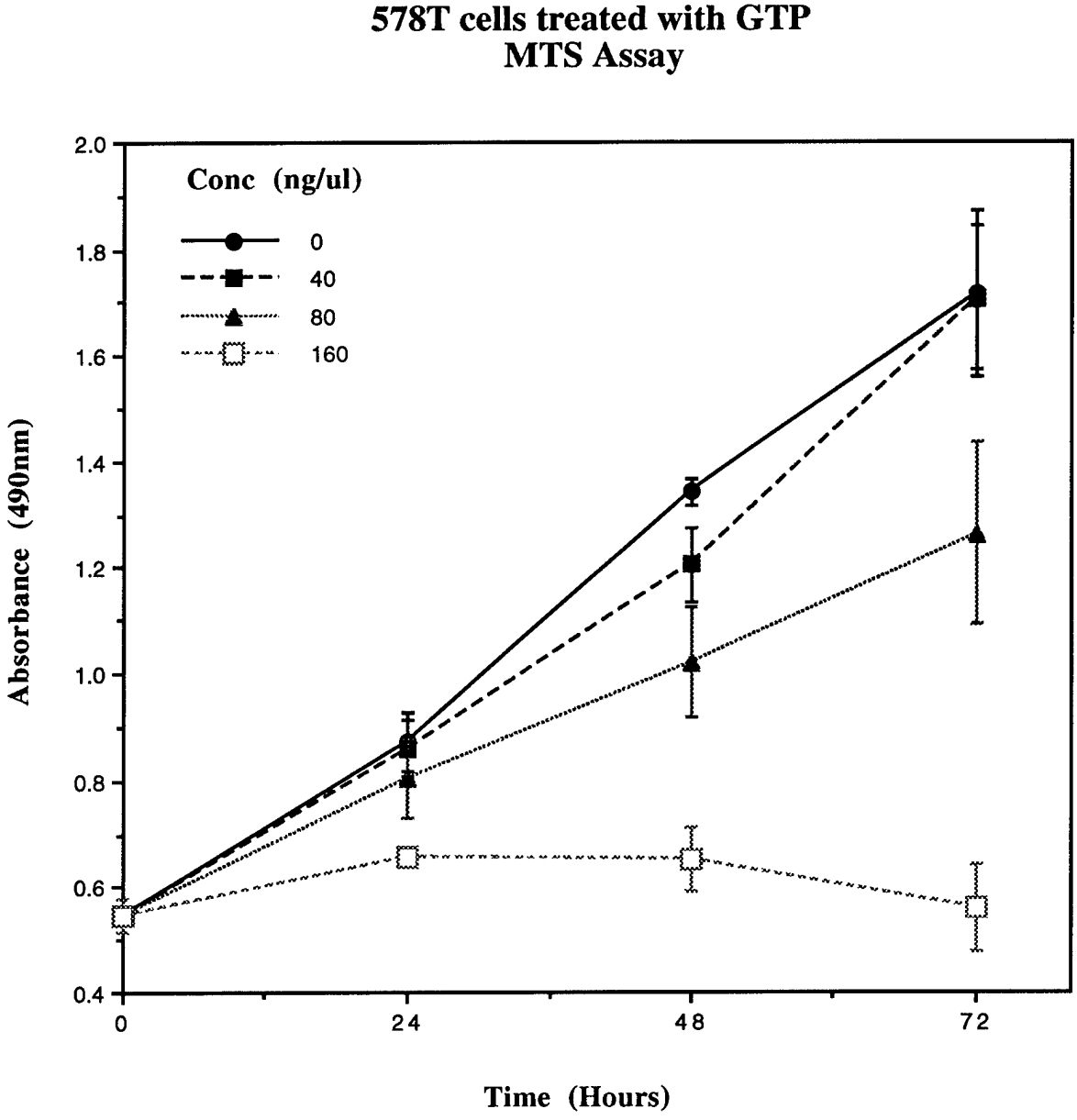

578T cells treated with GTP
MTS Assay
Conc (ng/ul)
0
40
80
160
Absorbance (490nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
24
48
72
Time (Hours)

Figure 2

EGCG treatment of 578T cells
MTS Assay

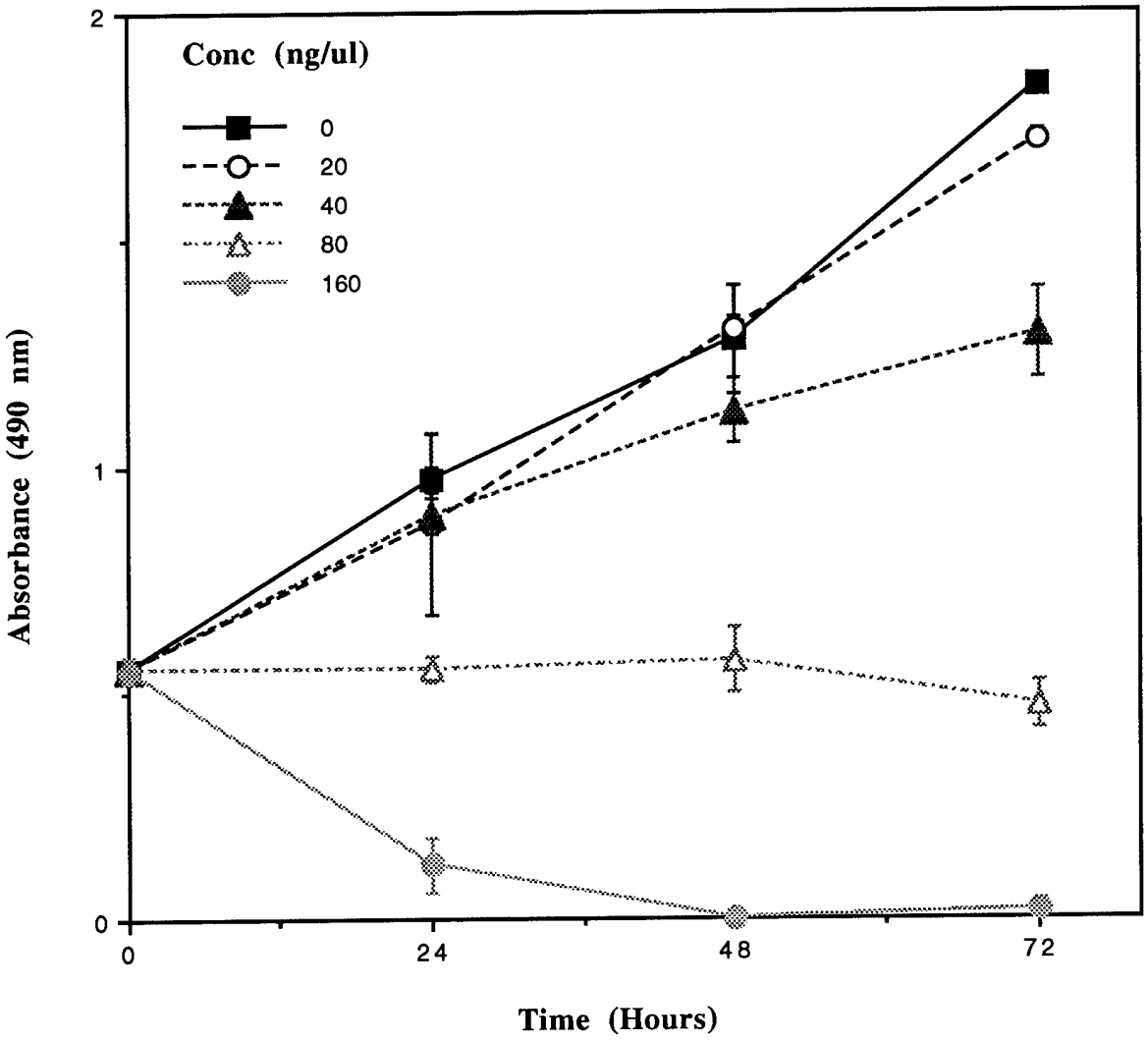


Figure 3

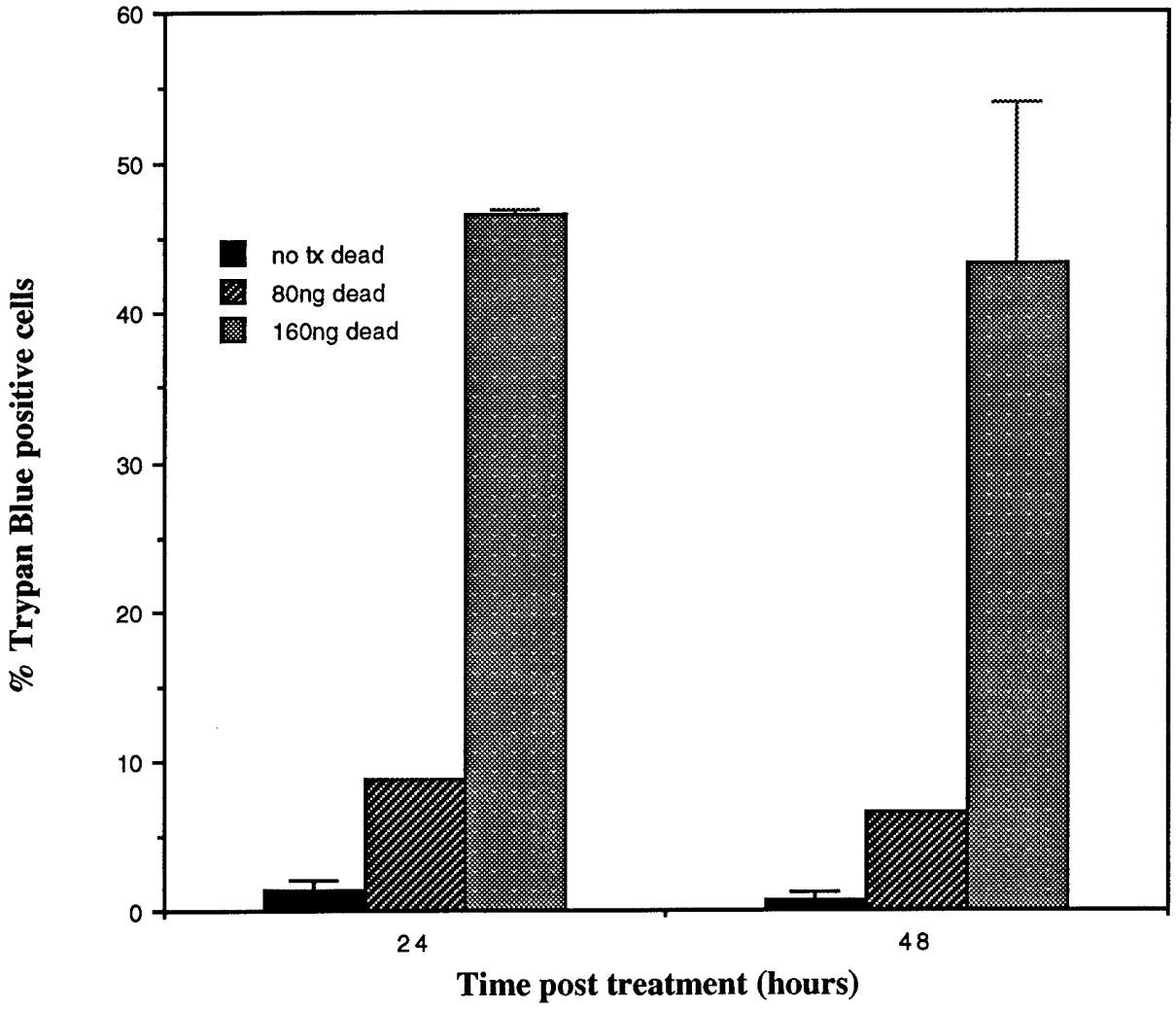
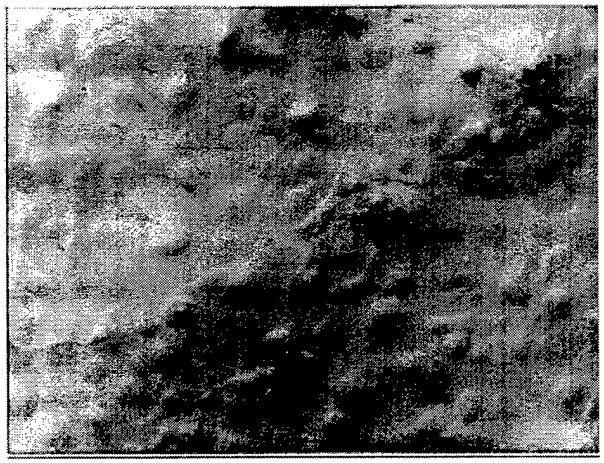

578T cells treated with EGCG
60
50
40
30
20
10
0
% Trypan Blue positive cells
no tx dead
80ng dead
160ng dead
24
48
Time post treatment (hours)

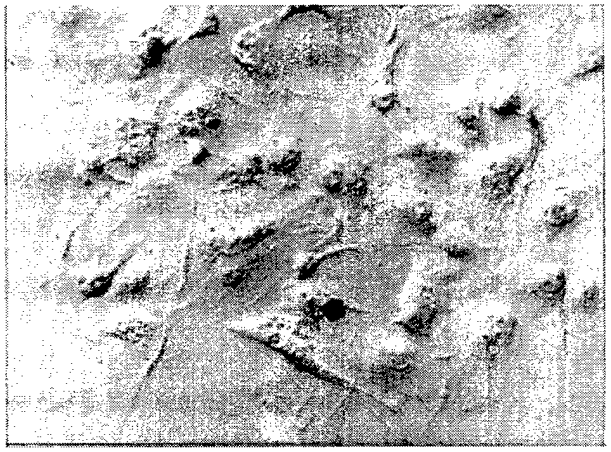
Figure 4

(A)



Cells treated with PBS vehicle only as control
Note the absence of any apoptotic bodies

(B)



Cells treated with 80 ng/ul EGCG for 48 hours
Black dots are the hallmark of apoptotic cell death.

Fig. 5 TUNEL Assay of Hs 578T cells

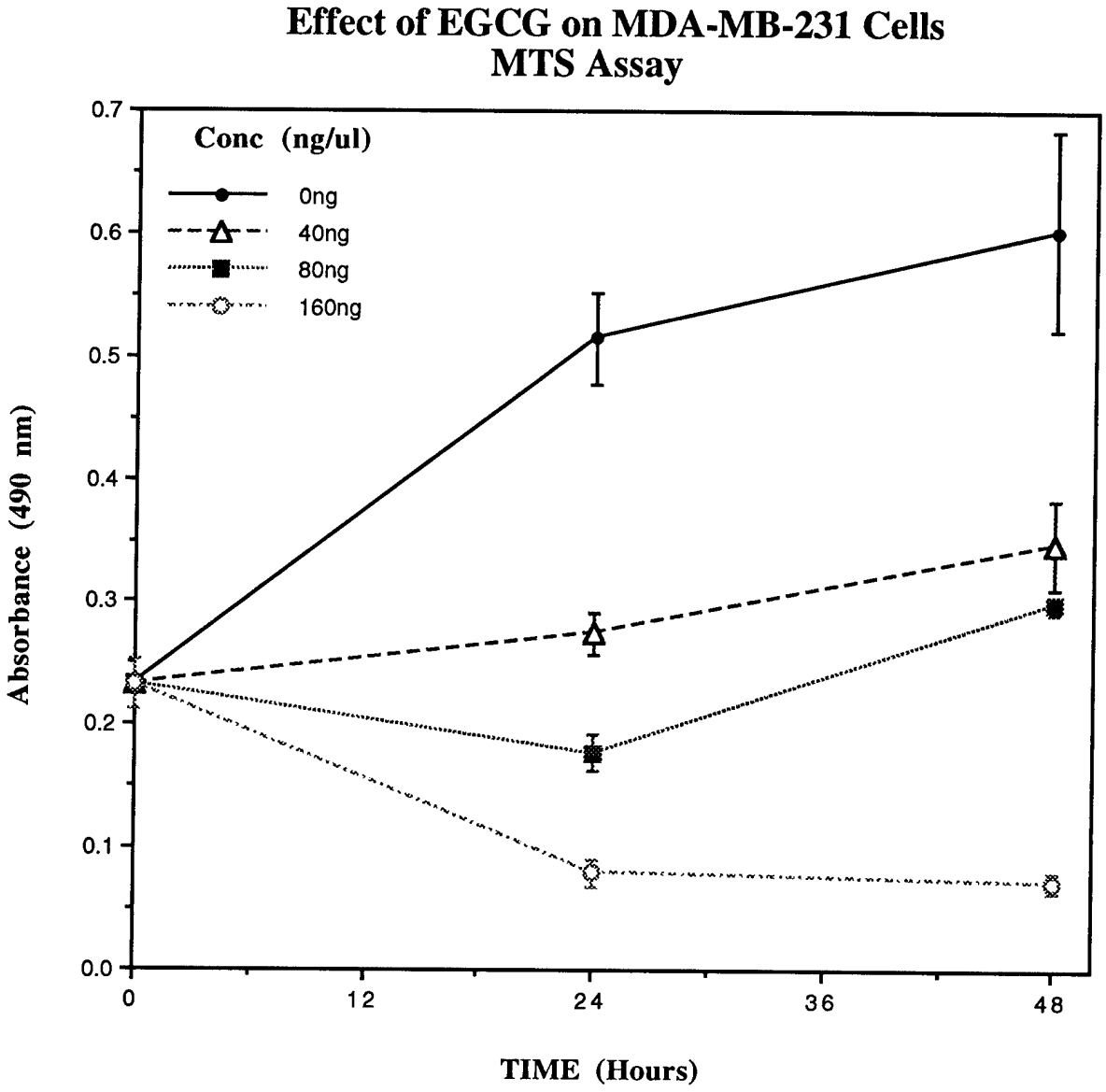

Effect of EGCG on MDA-MB-231 Cells
MTS Assay
Conc (ng/ul)
0ng
40ng
80ng
160ng
Absorbance (490 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
12
24
36
48
TIME (Hours)

Figure 6

Effect of EGCG on D3-1 cancer cells
MTS Assay

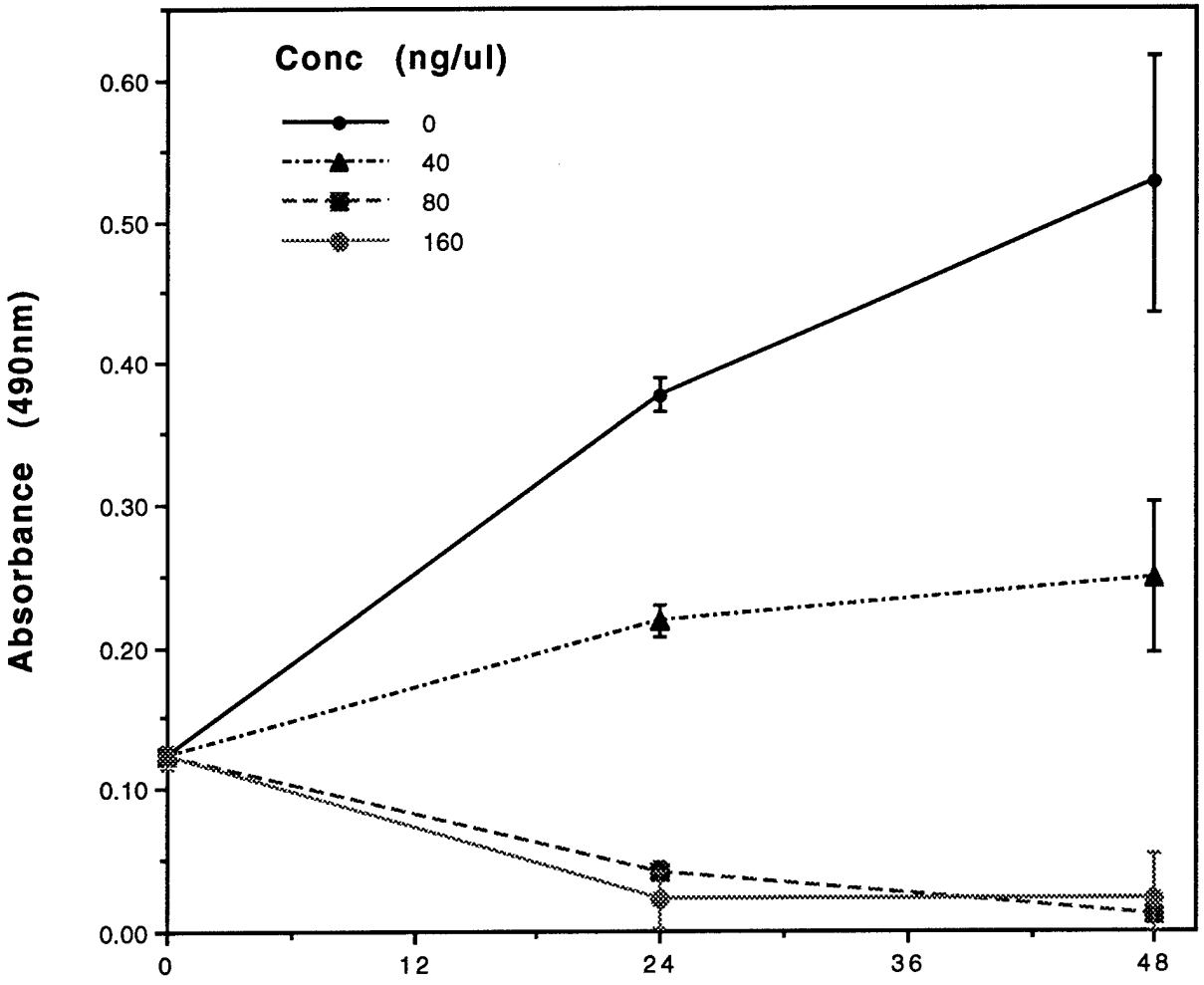


Figure 7

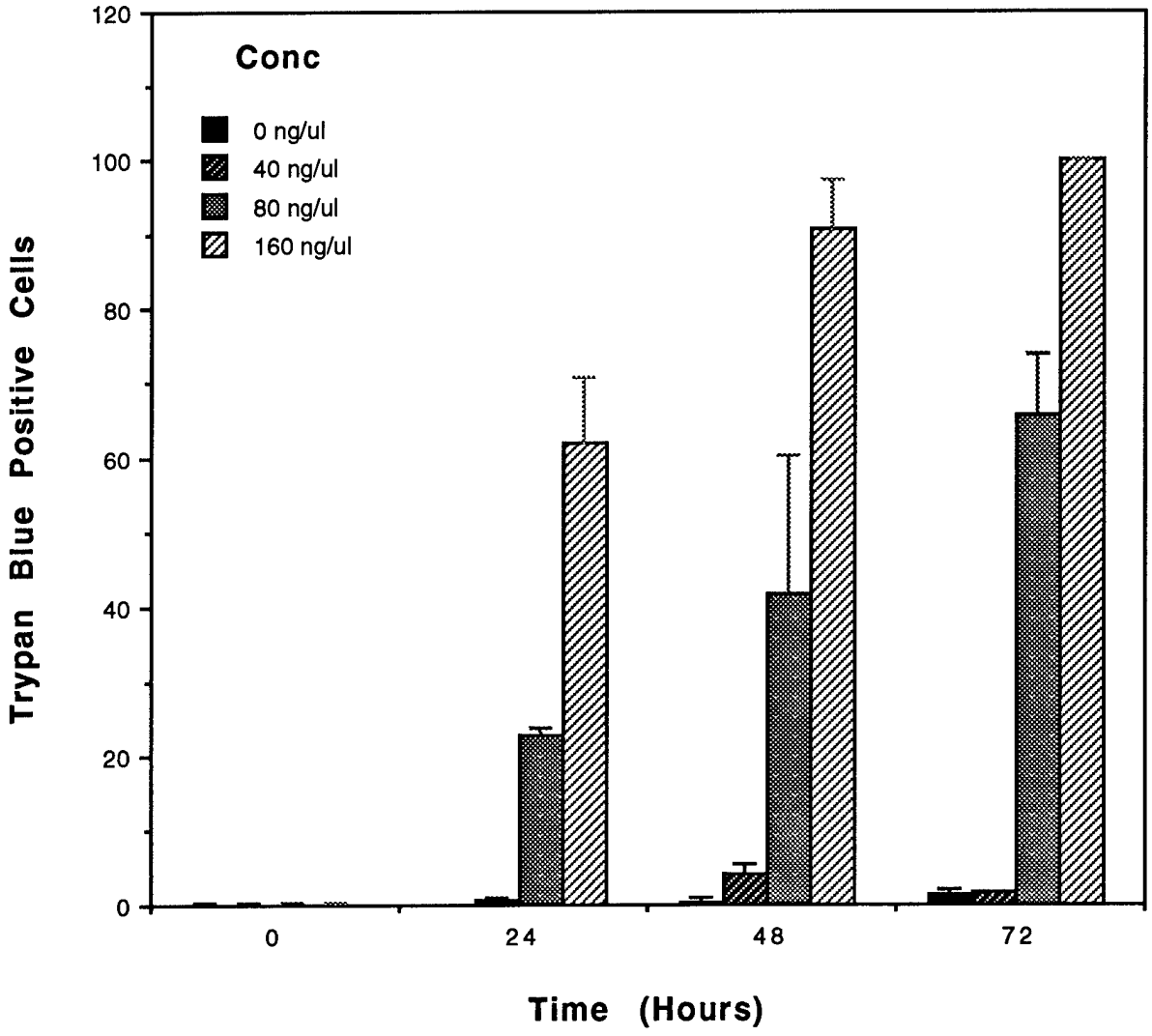

Conc
0 ng/ul
40 ng/ul
80 ng/ul
160 ng/ul
Trypan Blue Positive Cells
120
100
80
60
40
20
0
0
24
48
72
Time (Hours)

Figure 8

Effect of EGCG on MCF-7 Cancer Cells
MTS Assay

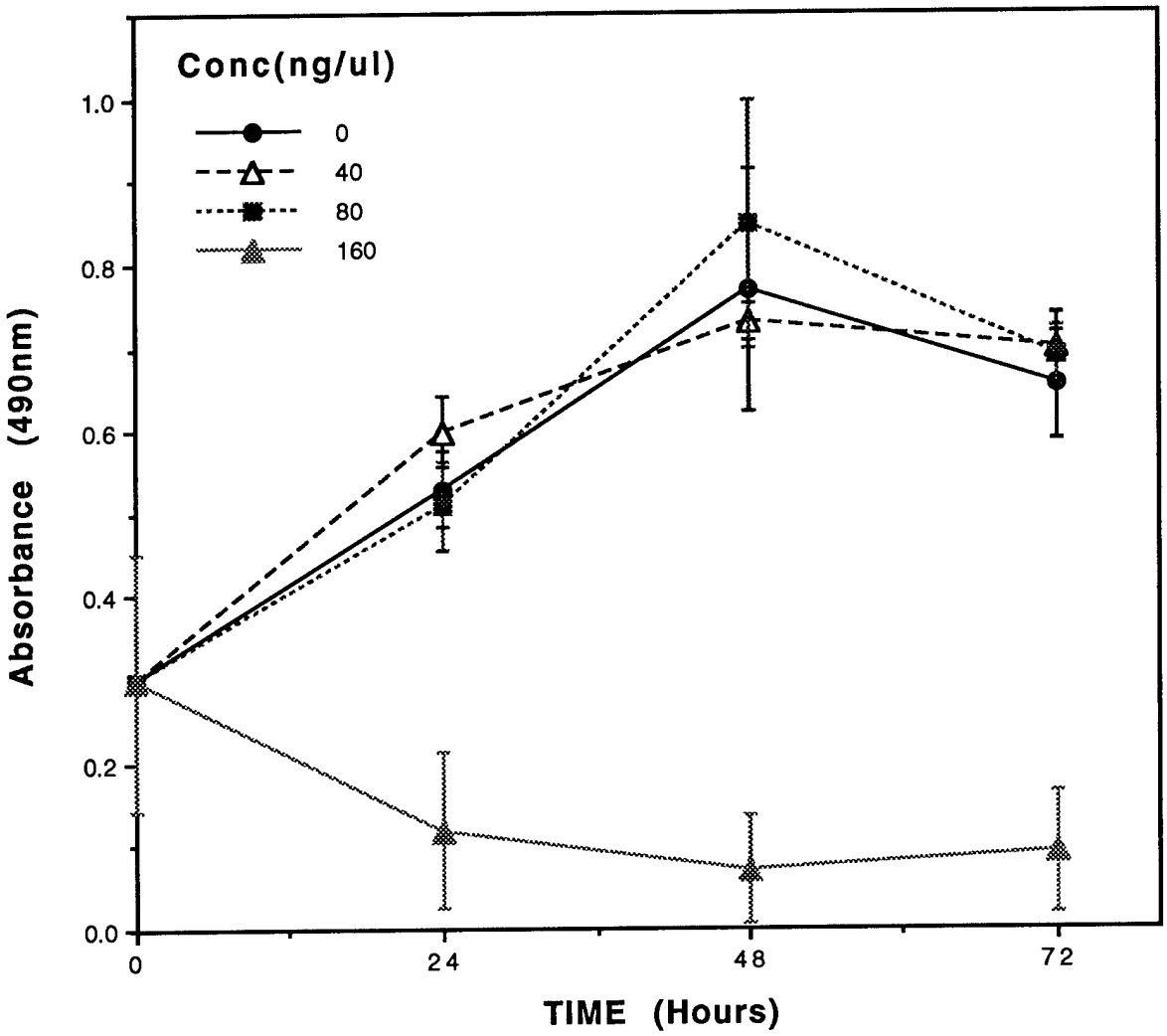


Figure 9

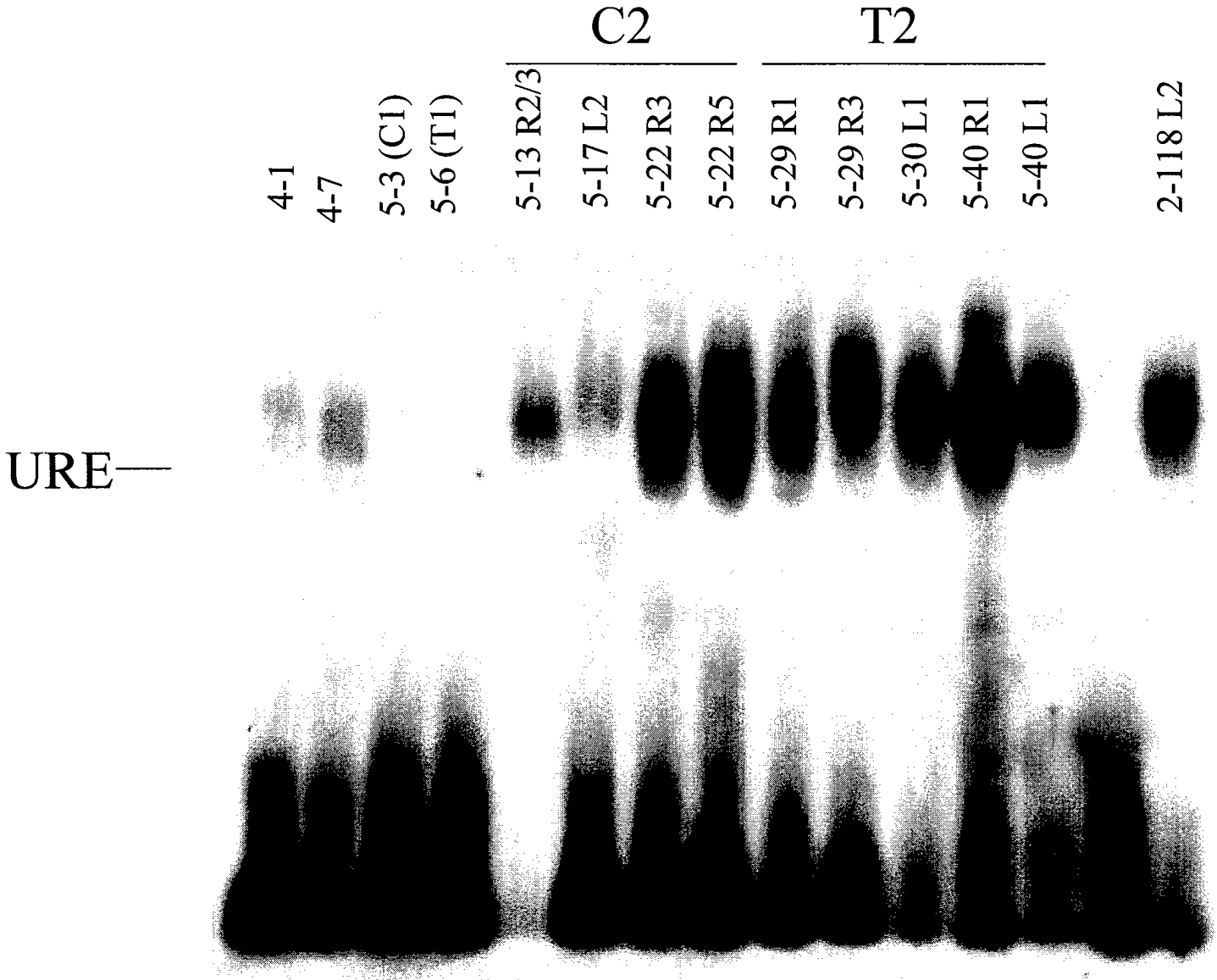


Fig. 10 Effect of Green Tea on NF-κB Binding Rat DMBA Tumor Tissue

C1 rats were fed water only, T1 rats were fed green tea only.C1 rats were fed water and were administered DMBA, T2 rats were fed green tea and were administered DMBA. 2-118L2 is a tumor expressing average NF-κB binding levels. 4-1 and 4-7 are normal mammary gland tissues expressing normal levels of binding for comparison.

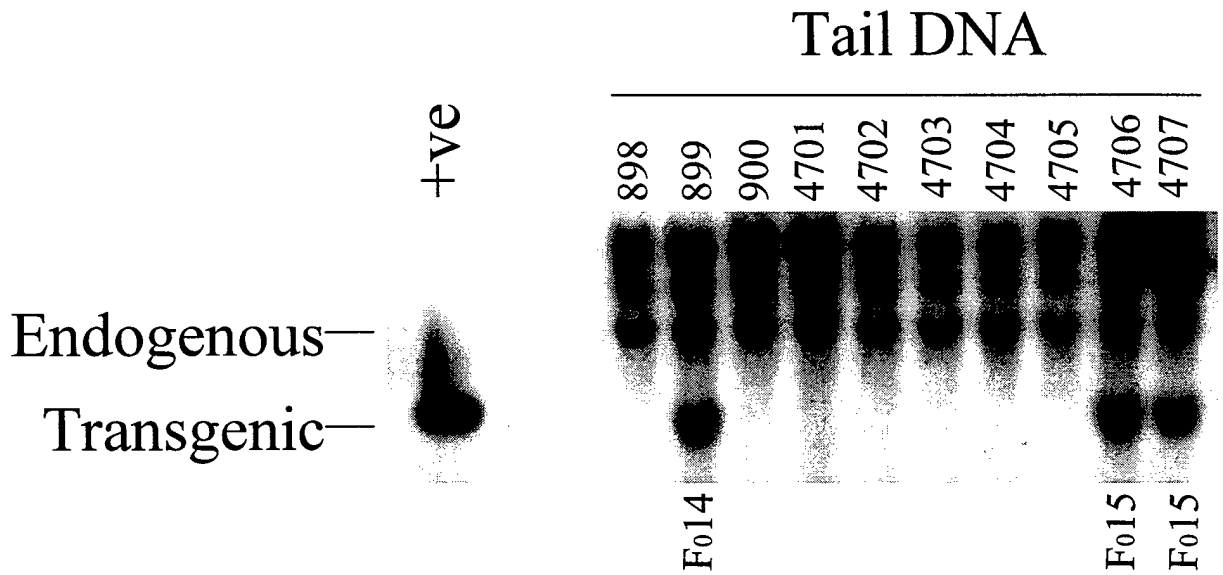


Figure 11. F1 Heterozygous Mice

MMTV-IκB-α 32/36 A construct digested with Bam H1 enzyme was used as a positive control (+ve). The MMTV- IκB-α 32/36 A construct was digested with Bam H1and 2kB fragment which corresponds to the insert was radioactively labelled and used as probe. 899 is from founder 14, 4706 and 4707 from founder 15.

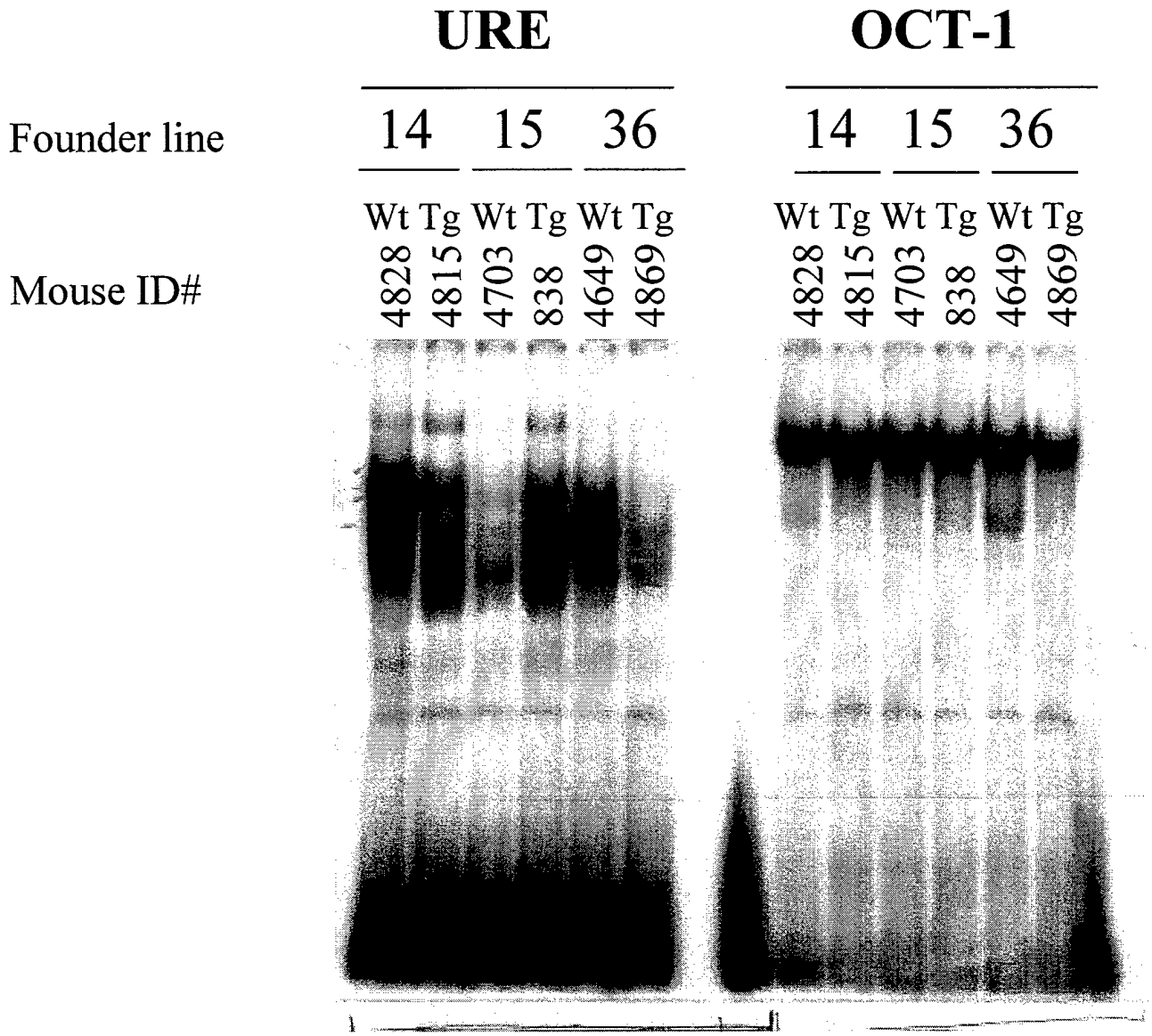


Figure 12 NF-kB/Rel binding activity in pregnant wild type and transgenic mice.